AD

TECHNICAL REPORT
Microbiology Series No. 20

A SURVEY OF SOME SPECIES OF ASPERGILLUS AND PENICILLIUM FOR PRODUCTION OF AFLATOXINS AND KOJIC ACID

by

F. W. Parrish, B. J. Wiley, E. G. Simmons, L. Long, Jr.

PIONEERING RESEARCH DIVISION

September 1965

U. S. Army Materiel Command
U. S. ARMY NATICK LABORATORIES
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FOREWORD

This report presents the results of a program of characterizing selected groups of fungi for the production of specific metabolites, namely, the aflatoxins and kojic acid. The aflatoxins were chosen for study because of the potential health hazard they represent and because of the ubiquitous occurrence in nature of the fungi which have been reported to produce them. Kojic acid was included in an attempt to extend our knowledge of the range of fungus species capable of producing this metabolite. A comprehensive review of the literature on the biogenesis, chemistry, microbiology, and assay of the aflatoxins is followed by details of the materials, methods, and results of the screening program.

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ABSTRACT

A total of 165 fungus strains representing 14 species of Aspergillus and 7 species of Penicillium were screened for production of aflatoxins and kojic acid in a defined medium. Aflatoxins were obtained only from two of the species of Aspergillus and from none of the species of Penicillium examined. Kojic acid was of more general occurrence, being obtained from strains of 9 species of Aspergillus and of 4 species of Penicillium.

A SURVEY OF SOME SPECIES OF *ASPERGILLUS* AND *PENICILLIUM* FOR PRODUCTION OF AFLATOXINS AND KOJIC ACID

I. INTRODUCTION

The Mycology Laboratory, Pioneering Research Division, which maintains the Natick Laboratories Culture Collection (QM), endeavors to contribute continually to the biological characterization of fungi in this collection. In view of the currently widespread interest in mycotoxins, particularly aflatoxins, and of our interest in the effects of fungi on material of use or of potential use to the Army, it was proposed to characterize all of the QM strains of *Aspergillus flavus* and of other species in the *A. flavus-oryzae* group as to their ability to produce aflatoxins and kojic acid. Additions to this basic group were to be made from other species of *Aspergillus* which have a history or reputation of toxicity or pathogenicity. Although most of the work was to be centered on species of *Aspergillus*, it was proposed to include selected strains of *Penicillium* species having a known ability to produce toxins in poultry feeds (24).

The history of the association of certain toxic symptoms with species of *Aspergillus* and the aflatoxins is relatively recent. For this reason it was believed that a critical review of the pertinent literature (1960 to date) would help to orient the viewpoints of the several mycological, chemical, and physical disciplines which were to contribute to the proposed study.

II. REVIEW OF THE LITERATURE

A. Origin of the Problem

In 1960, some 100,000 young turkeys died in the course of a few months from Turkey "X" disease (12). This disease proved to be non-infectious, and the possibility of poisoning was considered, but bacterial toxins, inorganic poisons, insecticides, and poisonous plants were not detected. A common factor in the outbreaks was the presence in the feed of groundnut (peanut) material from Brazil. Feeding experiments confirmed that the peanut material caused the symptoms (46).

Meanwhile, effects of this meal on other livestock, e.g., pigs and calves, were reported. Young animals, particularly ducklings, showed greater susceptibility than did adults (2), with acute or chronic

effects according to the animal species (31, 46). The liver was the affected organ, and a biological assay based on changes in liver tissues (41) was devised for the peanut toxin.

Work on extracts of peanut meal led to a chemical method for determining the presence and concentration of the toxin based on its blue fluorescence in ultraviolet light (42). In addition, it was suspected that the toxic substance might be a fungal metabolite, since a highly toxic sample of nuts from Uganda associated with the deaths of ducklings in Kenya was seen to be heavily contaminated with fungi. Pure cultures of some of the fungal species present in this sample were obtained, and from the mycelium of one of these isolates, grown 7 days at 27°C on Czapek's solution agar, a chloroform extract fluorescing blue in ultraviolet light was obtained. Day-old ducklings dosed with this extract died with the characteristic histological liver lesions of toxic peanut poisoning. The toxin producing fungus, Aspergillus flavus Link ex Fries (42), grows rapidly in mature peanuts harvested and stored under warm, humid conditions. Contaminated peanuts were obtained from Brazil, Kenya, Uganda, and India; but the fungus is ubiquitous, and the toxin has been found not only in peanuts but in grain products as well from other parts of the world (20).

Prior to the recognition of Turkey "X" disease and the isolation of the toxic agent (named "aflatoxin"), A. flavus had been implicated in other cases of animal poisoning. Burnside et al. (18) isolated a strain of A. flavus from moldy corn that was suspected of killing hogs, and Kulik (30) obtained material from peas infected with A. flavus which caused death when fed to cats and rabbits. In 1954, Paget described a noninfectious hepatosis of guinea pigs fed Bruce-Parkes Diet 18 which has been shown (38) to be due to aflatoxin formation in the peanut meal of this diet. Forgacs et al. (24) also isolated a strain of A. flavus from chicken feed while working on the relationship of mycotoxicoses to the poultry hemorrhagic syndrome.

The efforts of many scientists directed to the solution of various aspects of the aflatoxin problem have resulted in over 100 published papers to date. Aflatoxin was also the main topic in a symposium on "Mycotoxins in Foodstuffs" held at Massachusetts Institute of Technology in March 1964. Some of the results of these endeavors are reviewed here.

B. Bioassay for Aflatoxins

Several bioassays have been described using ducklings (6, 41), chick embryos (48), calf kidney cells (29), and embryonic lung cells (32).

Day-old Khaki Campbell (41) or Peking white ducklings (6) are fed toxic extracts. The degree of toxicity is estimated from the mortality rate together with examination of the livers for lesions resulting from the toxic effect.

The injection of toxic peanut extracts into embryonated hen's eggs results in the death of the embryo (39). The bioassay based on this observation (48) requires injection of the aflatoxin in propylene glycol into the yolk or, preferably, into the air cell before incubation. The embryo is observed over 21 days, and the toxicity is related to mortality. This assay is said to be more sensitive than the duckling test.

Aflatoxins inhibit incorporation of [^{14}C]leucine into protein in various liver preparations and induce vacuolation and destruction of monkey kidney cells (43). The toxicity of materials isolated from mold-infected peanut samples has been evaluated from the amount of cell destruction caused in calf kidney tissue cultures after 48 hours of incubation (29). Both the cytoplasm and nucleus of infected cells are destroyed.

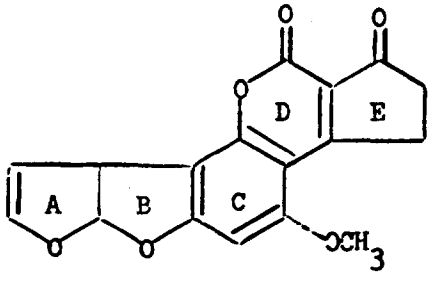
Aflatoxin B_1 and crude aflatoxin mixtures suppress mitotic division in heteroploid and diploid human embryonic lung cells (32). The effect is detectable after 4 hr and is maximal at 8-12 hr. As little as 0.01 μg of aflatoxin B_1 can be detected, and 0.03 μg produces 51% reduction in mitosis. In addition, synthesis of deoxyribonucleic acid from tritiated thymidine is inhibited, and normal cell morphology is affected.

C. Chemistry of Aflatoxins

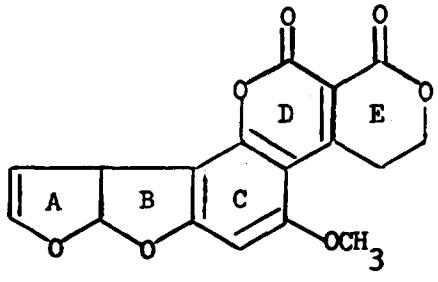
Chloroform extracts of highly toxic strains of A. flavus, when subjected to thin-layer chromatography (TLC) on silica-gel G with chloroform/methanol/formic acid (95:5:1 by volume), have been resolved into at least 12 clearly defined components which fluoresce under ultraviolet light (44). Four of the components designated B_1, B_2, G_1, and G_2, which cause liver lesions in ducklings, have been structurally elucidated. The letter B or G refers to the fluorescence color, blue or green, under ultraviolet light of 365 mμ. Aflatoxin B_1 was obtained in crystalline form and its physicochemical properties, including nuclear magnetic resonance (N. M. R.), infrared, and ultraviolet spectra, were determined by a group at the Unilever laboratories (49). Similar studies by researchers at Tropical Products Institute (T. P. I.), London, were made on crystalline aflatoxins B_1 and G_1; from elemental analyses and mass spectral data the molecular formulas $C_{17}H_{12}O_6$

and $C_{17}H_{12}O_7$, respectively, were deduced (36). The T. P. I. results for aflatoxin B_1 were confirmed by the Unilever group which also demonstrated the presence of a methoxyl group from the N. M. R. spectrum and a carbonyl function from the formation of a derivative with Girard T reagent (28). Further work by the T. P. I. group led to the isolation of crystalline aflatoxins B_2 and G_2, of diminished toxicity in comparison with B_1 or G_1 (25). Molecular formulas deduced from elemental analysis and mass spectral data indicated that B_2 and G_2 were dihydro-derivatives of B_1 and G_1, respectively. This inter-relationship was confirmed by hydrogenation of B_1 and G_1. From infrared and N. M. R. spectral data on the crystalline aflatoxin, certain structural features were proposed which are consistent with the complete structural assignments proposed by Asao et al. (8).

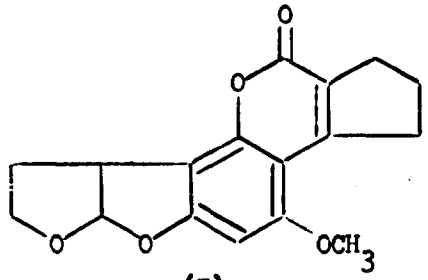
Aflatoxin B_1



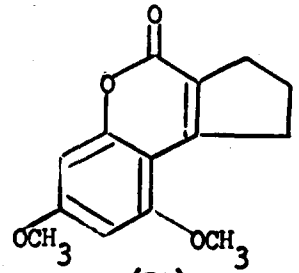
Aflatoxin G_1



Reduction of aflatoxin B_1 with 3 moles of hydrogen gave (I), the ultraviolet spectrum of which had a shape identical with that of synthetic coumarin (II), but all three maxima in the reduction product were

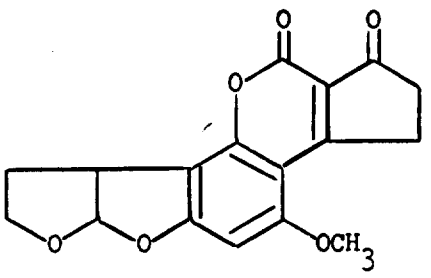


(I)



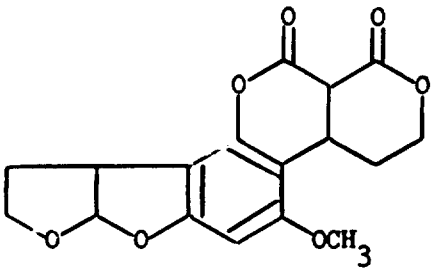
(II)

displaced to longer wave lengths by 7 mμ. The changes in the infrared absorption accompanying the reduction were explainable if the carbonyl group in the 5-membered ring of aflatoxin B_1 is cross-conjugated with the α, β-unsaturated lactone function. Examination of the N. M. R. spectrum of aflatoxin B_1 enabled the mode of attachment of the dihydrofuran ring to the coumarin nucleus to be determined. Comparison of the N. M. R. spectra of aflatoxins B_1 and G_1 led to the structure of the latter depicted above. Further work by Chang et al. (21) confirmed the structure of aflatoxin B_2 as dihydro-aflatoxin B_1 (25).



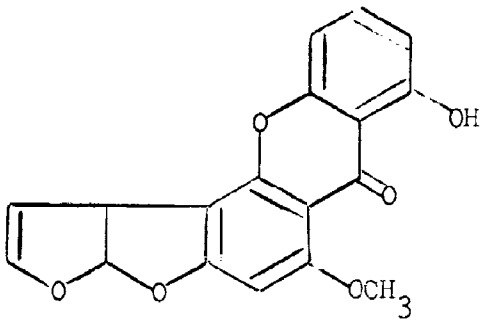
Aflatoxin B_2

It follows from the work of Hartley (25) and of Asao et al. (8) that aflatoxin G_2 has the structure:

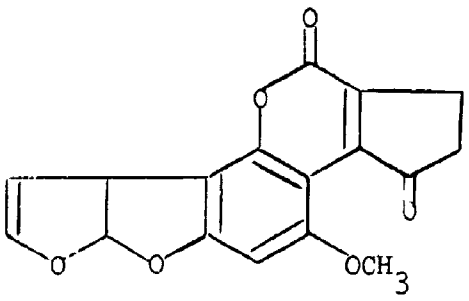


Aflatoxin G_2

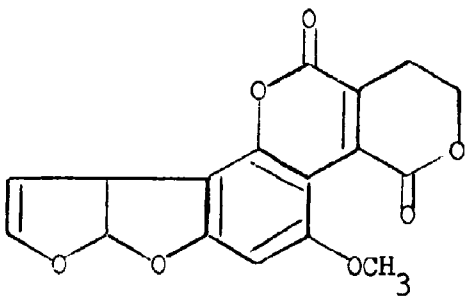
From similar considerations of spectral data of aflatoxins B_1 and G_1 compared with sterigmatocystin (17), van der Merwe et al. (33) have proposed different modes of attachment of the cyclopentenone structure of B_1 and of the unsaturated δ-lactone structure of G_1 to the coumarin nucleus.



Sterigmatocystin



Aflatoxin B_1

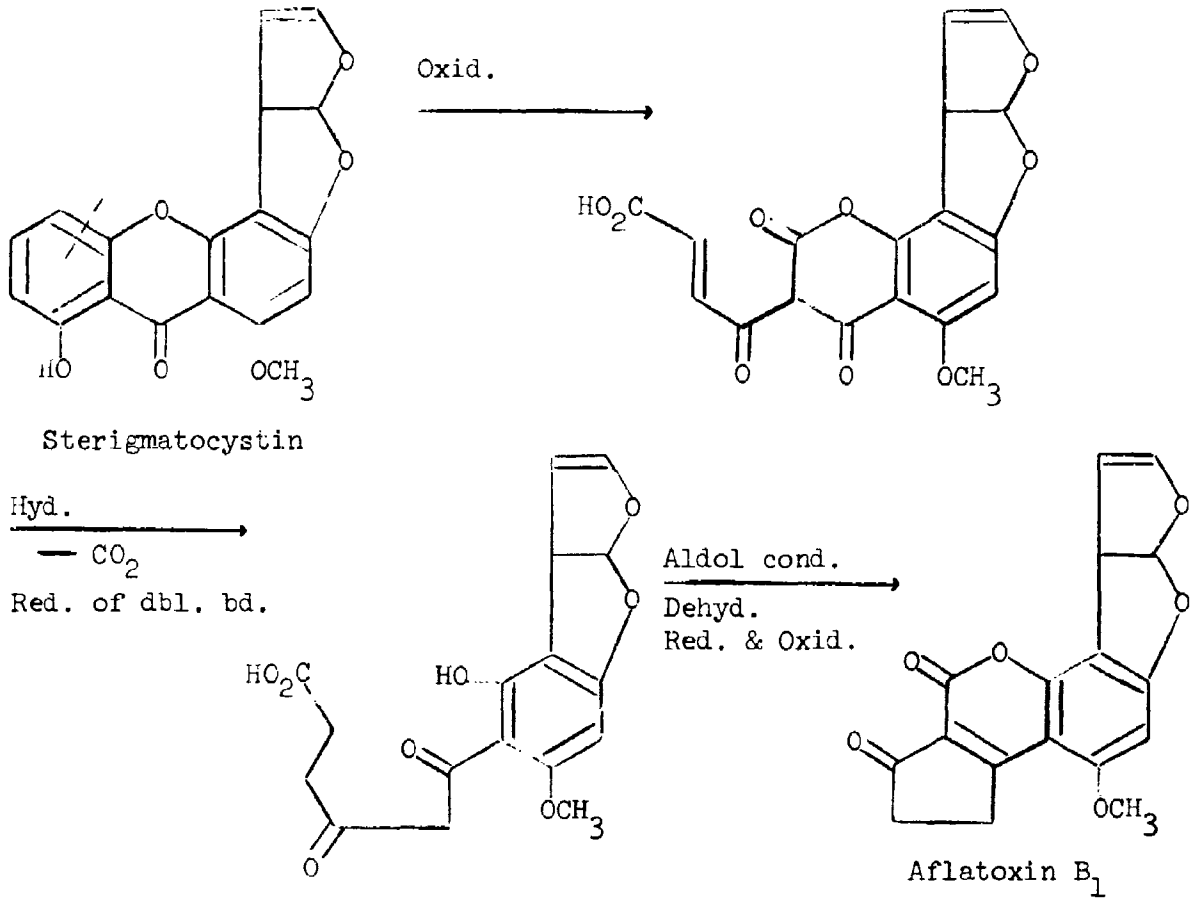


Aflatoxin G_1

X-ray diffraction studies of bromobenzene and bromothiophene solvates of aflatoxin G_1 (22) support the structure proposed by Asao et al. (8). An X-ray study of aflatoxin B_2 has also been made (45), and it confirms the structure proposed by Chang et al. (21).

D. Biogenesis of Aflatoxins

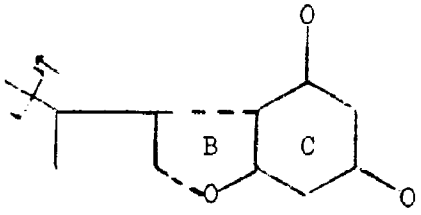
Holker and Underwood (27), in view of the structural identity of the bis-dihydrofuran moiety present in aflatoxin B_1 and sterigmatocystin, both of which are obtained as metabolic products from the genus Aspergillus, have proposed that both compounds are derived from a common biogenetic precursor or that sterigmatocystin is a precursor of aflatoxin B_1 as shown:



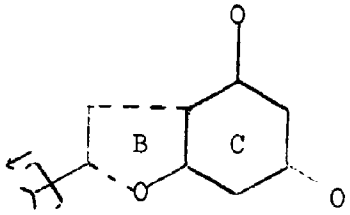
However, [^{14}C]sterigmatocystin was not incorporated into aflatoxin B_1 by either Aspergillus flavus or Aspergillus parasiticus Speare. Neither did these organisms produce labelled sterigmatocystin when grown on a medium containing [1-^{14}C]acetate. These results do not

rule out the possibility that the aflatoxins are derived from a precursor with a close structural resemblance to sterigmatocystin.

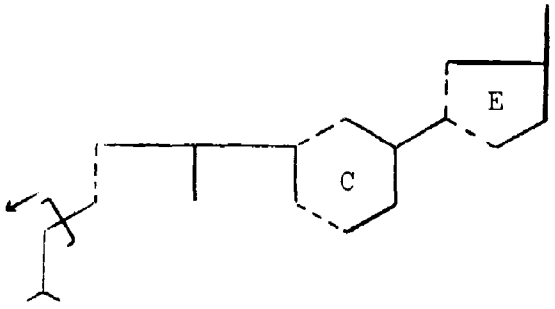
Moody considered the possibility that mevalonate is a precursor of aflatoxin (34). since the Acetate Rules did not lead to a plausible hypothesis for carbon skeleton formation. Skeletal structures derived partially from mevalonate (III, IV) or wholly from isoprene (V) were outlined:



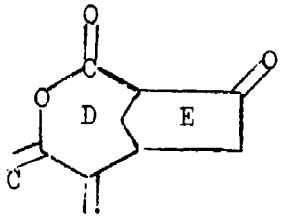
(III)



(IV)



(V)



(VI)

Formation of a β-substituted furan ring has a precedent in compounds related to limonin (5) whereas clerodin provides an analogy for subsequent formation of ring B (11). Conversion of skeleton (V) to the C, D, E ring system of aflatoxin B_1 might proceed by base-catalyzed scission of ring E of (VI) and reclosure in the alternative way.

The incorporation of labelled compounds into aflatoxins B_1 and G_1 by resting cell suspensions of A. flavus was studied by Adye and Mateles (1). Phenylalanine and tyrosine were incorporated, probably via a common intermediate, in agreement with the hypothesis that the coumarin

nucleus is derived from aromatic amino acids (16). Failure to incorporate shikimate and the poor incorporation of cinnamate and mevalonic lactone were attributed to difficulties of transport into the cell. The methyl group of methionine was incorporated, probably into the O-methyl group of the four aflatoxins. Acetate was incorporated, but no degradative studies were made to determine its position(s) and thus indicate the pathways involved. Tryptophan was incorporated relatively efficiently but its direct participation in coumarin synthesis is unlikely. A possible explanation suggested degradation of tryptophan to kynurenine which then gives anthranilic acid and alanine labelled in the 3-position. If the alanine were converted by transamination to [3-^{14}C]pyruvate, the presence of [2-^{14}C]acetate and its subsequent incorporation could be accounted for. Experiments testing these proposals have yet to be published.

E. Microbiology of Aflatoxins

Since the original identification of Aspergillus flavus as the fungus responsible for aflatoxin production in peanuts (42), numerous other strains of the species have been studied and about a dozen toxin producing strains of this species are maintained at the Commonwealth Mycological Institute (46).

In addition, Austwick and Ayerst (9) found that some of their toxin producing isolates were actually morphologically identical with Aspergillus parasiticus. It was thought that the dark colored spores of this species might be associated with toxin production, but subsequent examination of a number of toxin producing strains showed a wide range of morphological types often associated with the Aspergillus flavus-oryzae group. These strains grow and produce the toxin on a wide range of artificial media and appear to have no unusual specific requirements. Toxin is produced by the mycelium and most of the toxin is secreted into the medium. Trace amounts of toxin can be detected in the spores (10).

Subsequently the formation of aflatoxins by a strain of Penicillium puberulum isolated from moldy peanuts has been reported by Hodges et al. (26).

F. Toxin in Products Intended for Human Consumption

Liver and eggs from animals fed toxic peanuts do not contain aflatoxins in detectable amounts (3), and, although aflatoxin is present in the milk of cows fed toxic peanuts, bulking of milk supplies before distribution ensures dilution to harmless levels. Refined peanut oil,

which is used in margarine and cooking oil manufacture, is always toxin-free, even if produced from contaminated nuts, since aflatoxin in crude oil is removed by the alkali treatment of the refining operation. Edible peanuts are "hand picked selected" grade, and no sample of such material has yet been found to be toxic (46).

G. Prevention of Aflatoxin Formation

Aspergillus flavus is one of the so-called storage molds. The minimum moisture level for growth on the peanut kernel is 10% and the optimum ambient temperature is 30-35°C. Growth of the mold and toxin production are extremely rapid under tropical conditions, and peanuts which are uncontaminated at harvesting can contain detectable toxin within 48 hr (10). Generally the toxin occurs only in a small proportion of kernels. Attempts to detoxify kernels and meal have been unsuccessful, so it is apparent that solution of the problem lies in preventing mold attack during drying of the nuts. The moisture content of the kernels in the ground is at least 25%, but mold growth normally does not occur at this stage, possibly due to the physical barriers to hyphal penetration afforded by the intact shell and testa. The critical stage is that of post-harvest drying to below 10% moisture content which should be performed under good atmospheric conditions with harvesting practices that cause minimum damage to the shell. The moisture content of the kernels after harvesting must not rise above the critical level if A. flavus growth is to be prevented.

H. Assay Procedures for Aflatoxins

Aflatoxins are readily extracted from fungal cultures by chloroform (36, 49). Extraction from peanuts and peanut products is more complicated, mainly due to the need to remove fat from the extract before chromatographic examination. Extraction procedures have been compared (47), and rapid procedures have been described (19, 37).

A number of chemical and biochemical procedures have been proposed for the quantitative analysis of the isolated aflatoxins. The chemical methods generally involve chromatographic separation of the aflatoxin mixture on cellulose (7, 23), alumina (15, 26), or silica (44, 49). The concentrations of the separated aflatoxins are then determined either by visual comparison of their ultraviolet fluorescence with standards or by elution with methanol from the chromatographic adsorbent followed by measurement of the ultraviolet spectrum from 210 to 400 mμ. The spectrum provides a criterion of purity, and the aflatoxin concentration can be calculated from the absorbance at 363 mμ (25, 35).

A confirmatory test for aflatoxins involves spraying the chromatoplate with 50% sulfuric acid and observing the change in fluorescence color from blue or blue-green to yellow-green (44). The presence of aflatoxins B_1 and G_1 may be confirmed rapidly on less than 1 μg of isolated aflatoxin by treatment with trifluoroacetic acid, formic acid-thionyl chloride, or acetic acid-thionyl chloride. The reaction mixtures then are chromatographed on silica-gel G using 5% methanol in chloroform and the position of the fluorescent reaction product(s) compared with similarly treated standards. The reaction depends on addition of the acid to the vinyl ether group (4).

III. SCREENING OF QM FUNGUS ISOLATES

A. Materials and Methods

1. Cultures and growth conditions.

The 165 strains selected for examination represent 14 species of Aspergillus and 7 species of Penicillium (Table 1). Actively growing cultures 1-2 weeks old were used as sources of spore inoculum.

Erlenmeyer flasks of 250 ml capacity containing 50 ml of glucose-ammonium nitrate medium (GAN) or Czapek's medium (Cz), each supplemented with minerals (see Appendix), were inoculated, then incubated at 29 ± 1°C or 25 ± 2°C under stationary conditions for one week.

Toxin production was greater at both temperatures on the GAN medium than on the Cz medium. No significant difference in toxin production was observed on GAN medium at these temperatures; the lower temperature (25 ± 2°C) was selected for subsequent experiments.

Growth conditions for aflatoxin production were not investigated extensively since no unusual specific requirements have been demonstrated.

2. Extraction procedure.

At the end of the one-week growth period, a volume of chloroform equal to that of the culture medium was added to each flask; the flask and its contents were heated and magnetically stirred until the chloroform vapor penetrated the cotton plug. The mixture was cooled to room temperature, then filtered through glass wool into a separatory funnel; the mycelial mat was discarded. The filtered mixture was shaken, the chloroform layer was removed, and the aqueous layer was re-extracted with an equal volume of chloroform. These extractions were sufficient to remove the aflatoxins from the mycelium and the culture medium.

The combined chloroform extracts were dried with anhydrous sodium sulfate, filtered, and evaporated to dryness under reduced pressure.

3. Assay procedure for aflatoxins.

The residue which remained after evaporation of the chloroform was dissolved in a known volume of chloroform (0.1-0.5 ml) and a sample (5-20 µl) was examined by thin-layer chromatography (TLC) on silica-gel G with development by means of 2% methanol in chloroform.

The developed plates were examined under a 365 mµ ultraviolet light. Aflatoxins, when present, appeared as blue or blue-green spots at R_f 0.4-0.5. The presence of aflatoxin was confirmed by spraying the plates with 50% sulfuric acid and observing the color change under ultraviolet light.

Extracts of the 30 aflatoxin producing strains were examined on 250µ thick silica-gel G plates triple-developed with 2% methanol in chloroform to separate the four major components. The separated aflatoxins B_1, B_2, G_1, and G_2 were estimated by visual comparison with standard amounts of controls (Table 2). The individual controls were obtained by preparative TLC of a crude aflatoxin mixture (kindly supplied by Dr. G. N. Wogan, Department of Nutrition, Food Science and Technology, Massachusetts Institute of Technology) and were identified by their ultraviolet and infrared spectral characteristics. The concentrations of the control solutions used in TLC were determined from their optical density at 363 mµ (see Appendix).

4. Assay procedure for kojic acid.

Thin-layer chromatography of samples of the chloroform extracts was performed on silica-gel G with a solvent composed of benzene/acetic acid/methanol (3:1:1). The plates were then sprayed with an aqueous solution of 0.07 M ferric chloride to detect kojic acid (40), the amount of which was described as weak, medium, or strong as evidenced by the amount of red color produced (Table 1).

Kojic acid, isolated from the culture medium by chloroform extraction, was identified by comparison of its melting point and infrared spectrum with those of authentic material.

Spectrophotometric determination of kojic acid also was made using its reaction with ferric chloride. This assay was performed on a sample of the chloroform extract since it cannot be made directly on the GAN culture medium. The sample containing 0.0-6.0 mg of kojic acid was treated with 1 ml of 0.37 M ferric chloride in 0.1 N hydrochloric acid, its volume was made up to 25 ml with water, and its absorbance was read at 500 mµ. The absorbance of kojic acid-ferric chloride solution

at 500 mμ obeyed Beer's law, and the molar absorptivity was 1240. From culture medium containing up to 12 mg/ml of kojic acid, 2.5% of the kojic acid was isolated by the two extractions with chloroform.

B. Results and Discussion

A chemical assay of the extracted aflatoxins seemed preferable to any of the various bioassays by virtue of simplicity, speed, and reproducibility. In agreement with the findings of Trager et al. (47), better separation of aflatoxins was obtained by TLC on silica-gel than on alumina. Thin layers of cellulose required longer development times with solvents than did silica; in addition, the former adsorbent is not suitable for detecting other metabolites by charring with sulfuric acid. Chromatography on kieselguhr (1) did not give better resolution in our hands than that obtained with silica-gel.

Visual comparison of chromatographed samples with standards of known concentration gave a rapid, semiquantitative determination of the aflatoxins B_1, B_2, G_1, and G_2 suitable for our survey work. A more accurate determination could be obtained from the absorbance at 363 mμ of methanolic solutions of the eluted aflatoxins (35).

On spraying developed chromatoplates of aflatoxin-containing samples with sulfuric acid, the change in fluorescence to yellow-green (44) invariably was observed.

Aflatoxins were produced by 26 of 93 strains of Aspergillus flavus and by all of the 4 strains of A. parasiticus examined. Aflatoxins were not produced by any strains of the 12 other species of Aspergillus used in this study, nor were they obtained from any of the 17 strains of Penicillium which included 5 strains of P. puberulum (Table 1).

Kojic acid was produced by all but one of the same 93 strains of A. flavus and by all of the 4 strains of A. parasiticus as well as by miscellaneous isolates of other Aspergillus species. Six of the 17 strains of Penicillium examined also produced kojic acid (Table 1).

Three Aspergillus flavus strains (QM 3352, QM 3480, QM 4780) which produced aflatoxins 2-3 days after inoculation gave no indication of kojic acid production after one week's incubation. These were retested over a period of 1-17 days; it was found that kojic acid was produced by them after 8-10 days of incubation (Table 2).

Table 1. Production of Aflatoxins and of Kojic Acid by Species of Aspergillus and Penicillium

Species	No. of strains tested	Strains producing aflatoxins	Kojic Acid Production*			
			None	Weak	Medium	Strong
A. chevalieri	1	0	1			
A. clavatus	4	0	2	2		
A. effusus	1	0				1
A. fasciculatus	2	0	2			
A. flavus	93	26	1	35	28	29
A. flavus-oryzae group	5	0		3	1	1
A. fumigatus	9	0	7	2		
A. fumigatus m. helvola	1	0	1			
A. fumigatus v. minimus	1	0	1			
A. micro-virido-citrinus	1	0	1			
A. nidulans	11	0	9	1	1	
A. nidulans group	2	0	2			
A. nidulans v. acristatus	1	0	1			
A. nidulans m. alba	1	0	1			
A. nidulans v. echinulatus	2	0	2			
A. niger	1	0	1			
A. oryzae	3	0	1		2	
A. parasiticus	4	4		1		3
A. rugulosus	1	0	1			
A. tamarii	3	0		3		
A. ustus	1	0				1
P. albidum	1	0	1			
P. citrinum	5	0	2		3	
P. citrinum series	1	0	1			
P. estinogenum	1	0	1			
P. griseo-fulvum	1	0				1
P. puberulum	5	0	5			
P. purpurogenum	1	0			1	
P. rubrum	2	0	1	1		

* Weak: up to 0.25 mg/ml; Medium: 0.25-1.0 mg/ml; Strong: above 1.0 mg/ml.

Table 2. Production of Aflatoxins and of Kojic Acid by Strains of *Aspergillus flavus* and *A. parasiticus*

QM No.	Species	Aflatoxins in units/ml* B_1	B_2	G_1	G_2	Kojic Acid
4m	A. flavus	10	4	6	2	weak
63c	A. flavus	20	8	30	8	weak
118a	A. flavus	4	2	2	0	medium
132g	A. flavus	4	2	0	0	medium
134k	A. flavus	2	0	2	0	weak
305	A. flavus	4	2	4	2	medium
870	A. flavus	2	2	0	0	strong
931	A. flavus	4	2	0	0	weak
2675	A. flavus	4	0	0	0	medium
3127	A. flavus	10	4	2	0	weak
3352	A. flavus	60	20	40	4	strong
3371	A. flavus	30	10	30	10	strong
3447	A. flavus	4	2	10	4	medium
3455	A. flavus	30	10	30	10	weak
3469	A. flavus	8	6	0	0	strong
3480	A. flavus	10	4	6	2	strong
3556	A. flavus	20	8	0	0	strong
3569	A. flavus	10	6	0	0	weak
3614	A. flavus	4	2	10	8	strong
3955	A. flavus	2	0	2	0	medium
4780	A. flavus	60	20	60	20	medium
6738	A. flavus	10	6	4	2	medium
6739	A. flavus	2	0	0	0	strong
7671	A. flavus	20	8	0	0	medium
8361	A. flavus	4	2	2	2	weak
8580	A. flavus	4	2	4	2	medium
883	A. parasiticus	20	8	30	8	strong
884	A. parasiticus	20	8	30	8	strong
6736	A. parasiticus	40	20	60	20	weak
8378	A. parasiticus	10	2	10	2	strong

*Unit of: B_1 = 0.051 µg; B_2 = 0.018 µg; G_1 = 0.012 µg; G_2 = 0.011 µg.

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APPENDIX

Czapek's solution (Cz)*

Glucose	50.0 g
$NaNO_3$	2.0 g
KH_2PO_4	1.0 g
KCl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Water	1000.0 ml
Mineral supplement	2.0 ml

Glucose ammonium nitrate sol. (GAN)*

Glucose	50.0 g
NH_4NO_3	2.4 g
KH_2PO_4	10.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
Water	1000.0 ml
Mineral supplement	2.0 ml

Mineral supplement solution*

$FeSO_4 \cdot 7H_2O$	1.0 g
$CuSO_4 \cdot 5H_2O$	0.15 g
$ZnSO_4 \cdot 7H_2O$	1.0 g
$MnSO_4 \cdot H_2O$	0.1 g
$K_2MoO_4 \cdot 5H_2O$	0.1 g
0.25 N HCl	100.0 ml

Before use, the mineral supplement solution (1 volume) is diluted with water (9 volumes).

Determination of aflatoxin concentration on the basis of ultraviolet absorbance at 363 mμ**

$$\text{Aflatoxin concn } (\mu g/\mu liter) = \frac{\text{Absorbance at 363 m}\mu \text{ X mol wt}}{\text{Molar absorptivity X path length (cm)}}$$

Aflatoxin	Mol. Wt.	Molar Absorptivity**
B_1	312	22,000
B_2	314	23,400
G_1	328	18,700
G_2	330	21,000

* Ref: 13, 14
** Ref: 25, 35

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13 ABSTRACT

A total of 165 fungus strains representing 14 species of Aspergillus and 7 species of Penicillium were screened for production of aflatoxins and kojic acid in a defined medium. Aflatoxins were obtained only from two of the species of Aspergillus and from none of the species of Penicillium examined. Kojic acid was of more general occurrence, being obtained from strains of 9 species of Aspergillus and of 4 species of Penicillium.

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Kojic Acid	2		2			
Fungi	2		1			
Aspergillus	1		1			
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